Diagnosis of Cancer using MAS

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Introduction

The MR spectroscopy (MRS) method gives a comprehensive window into tissue biochemistry and interrogates cancer tissue for diagnostic and prognostic markers. In vivo MRS adds to MRI information when describing tissue abnormalities in patients. For tissue specimens, studies have shown that ex vivo MRS can characterize tumors (1-3). Conventional MR spectroscopy of biopsies has been shown to classify cancer tissue from cervix (4,5) and breast (6) cancer patients.

Increased spectral resolution gives more detailed metabolic information than conventional MRS analysis. High resolution magic angle spinning (HR MAS) MRS has been used to study intact tissue specimens since 1996 (7). HR MAS MRS is a high-throughput technology with the potential of becoming fully automated. It has a high degree of reproducibility, and its non-destructive nature allows specimens to be evaluated by microscopy after spectral analysis, making direct comparisons to morphologic characteristics feasible. Findings from brain (8-11), cervical (12,13), breast (14), and prostate cancer (15-18) have proven MAS as a promising tool in cancer diagnosis and treatment monitoring.

High-resolution MAS MRS studies are still performed in research programs, however, clinical studies have started to explore the value of the method for clinical diagnosis and treatment monitoring. The objectives of this presentation are to describe the HR MAS MRS techniques used for cancer specimens, and present some results that can be obtained in diagnosis of different cancer types.

Clinical Motivation

Despite research efforts and identification of many putative good prognostic factors, few of them are proving clinically useful for identifying patients at minimal risk of relapse, patients with a worse prognosis, or patients likely to benefit from specific treatments. Adjuvant chemotherapy and hormonal treatment improve survival for breast cancer patients but have potentially serious side effects, and are costly. Because adjuvant treatment should be given to high-risk patients only, and traditional prognostic factors as lymph node status and tumor size are insufficiently accurate, better or supplementary predictors of high-risk and treatment response are needed. Several new experimental methods in addition to MR determined metabolic pattern are being explored to improve diagnostic and prognostic information. These methods comprise among others, immunohistochemistry, gene expressions arrays, and protein arrays. Successful diagnosis and cancer treatment of the future are being developed with a focus on the molecular targets underlying the pathophysiology of neoplasia. These targets might be defined on the basis of genetic, protein and metabolic techniques, which define targets expressed as a result of a tumor's differentiation state or tissue of origin; or targets mediating drug uptake or metabolism (19).

Correlation of MR spectra to patient diagnosis and histopathology have been established by conventional MR spectroscopy of intact tissue samples (2,5,6). However, spectral resolution in such spectra is low and the biochemical information thereby limited. MR spectra of cell or

tissue extracts provide detailed information on chemical composition, but at the cost of tissue destruction and possibly modified composition. An advantage of HR MAS tissue analysis is the possible translation to in vivo MRS examination of patients. Specific metabolic features found in tissue analyses might be mapped in vivo using single volume or spectroscopic imaging techniques.

High-resolution magic angle spinning (HR MAS)

Tissue can be considered as a semisolid giving broad line in *ex vivo* spectra obtained by conventional MR spectroscopy. The lack of molecular mobility leads to anisotropic interactions, imposing a spin orientation dependence on the MR frequency (20). Anisotropic interactions consist of direct homonuclear and heteronuclear magnetic dipolar interactions, indirect electron coupled interactions, electric quadrupolar interactions and electron shielding interactions.

Andrew *et al*. (21) and Lowe (22) first described the narrowing of MR lines when solids were spun at the magic angle. Line broadening in solids can be reduced by spinning the sample rapidly about an axis inclined 54.7° to the direction of the static magnetic field (Figure 1). The spinning splits the broad resonance into a narrow line at the isotropic resonance frequency and spinning sidebands (23). All spin interactions become time-dependent and sidebands appear at integer multiples of the spinning rate. The time independent part of the anisotropic interactions is dependent on $(3\cos^2\theta-1)$ and is cancelled by the choice of angle. The time dependent anisotropic interactions average over a rotation period. If the spinning rate is much larger than the anisotropic spin interaction, the sidebands are well separated from the central line and their intensity decrease with increasing spinning rate. As a consequence, anisotropic interactions are averaged to their isotropic value, resulting in substantial line narrowing.

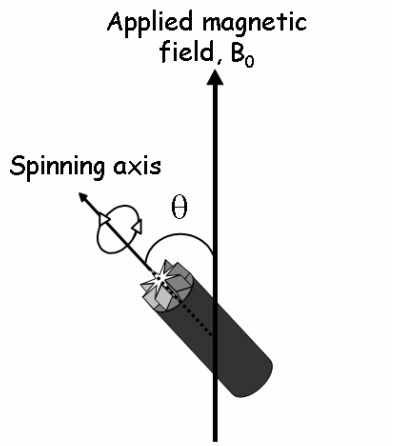


Figure 1: Schematic presentation of a sample in a magic angle spinning probe. θ is the magic angle, 54.7 °, B_0 is the static magnetic field.

Although narrower lines are obtained because of the MAS effect, large molecules like proteins and lipids appear as broad signals in the HR MAS spectrum. A common method to reduce these broad signals is by utilizing their short T_2 relaxation times. Suppression of signals with short T_2 values can be performed using a spin-echo sequence with long echo times for acquisition (24).

Metabolites detected with 1H MAS MR spectroscopy in cancer specimens

Biological samples, such as cancer tissue, comprise a vast amount of MR detectable compounds and the resulting high field proton MR spectra can be very complex (Figure 2).

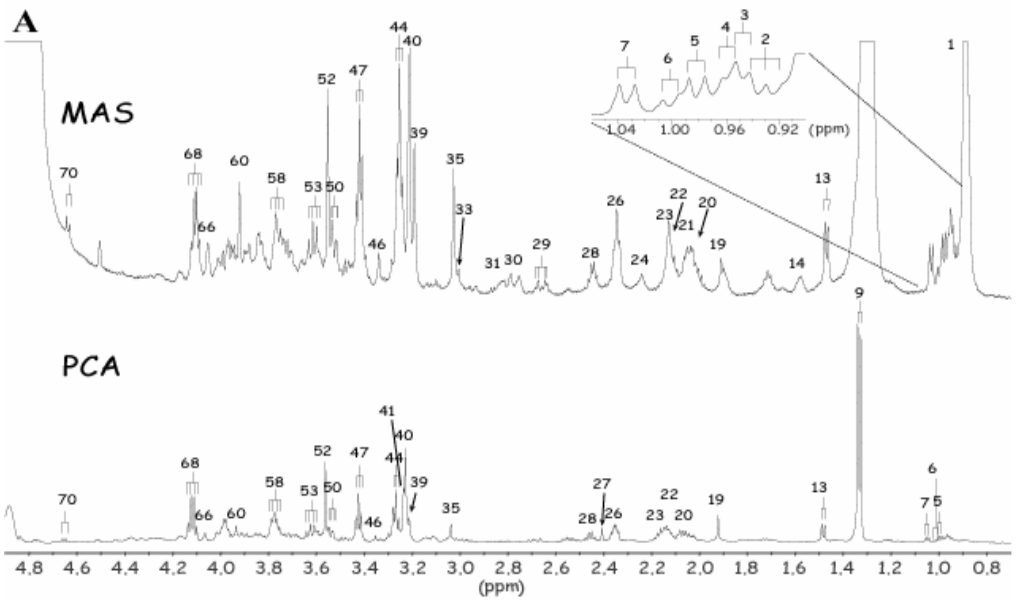


Figure 2: The resolution of MAS spectra from intact breast cancer tissue (A) is comparable to spectra of PCA extracts (B) from breast cancer specimens. Some of the assigned metabolites are: $-CH_3$ fatty acids (1), lactate (9, 68), $-\mathbf{CH_2}-CH_3$ fatty acids (10), creatine (35, 60), choline (40), phosphocholine (41), glycerophosphocholine (42), taurine (44, 47), *myo*-inositol (45, 50, 53), scyllo-inositol (46) and glucose (70). For complete assignments please see reference (Sitter B et al. NMR in Biomed 2002). The spectra were acquired at a Bruker AVANCE DRX600 instrument. The MAS spectrum was obtained using a spin echo sequence with an echo time of 285 msec.

Assignment of signals in HR MAS spectra are performed using published data, two-dimensional MR-techniques such as COSY and J-resolved spectroscopy, and spiking of samples. The latter method may be inaccurate in tissue samples, since the metabolites and authentic standards can be in different compartments and therefore give rise to slightly different chemical shifts. Several papers have attempted to assign signals in HR MAS spectra of cancer, including breast cancer (25), high grade gliomas (9), prostate cancer (18) and cervical cancer (12).

The effect of using a spin-echo sequence for acquisition of spectra to reduce contribution of broad signals can be seen in Figure 3.

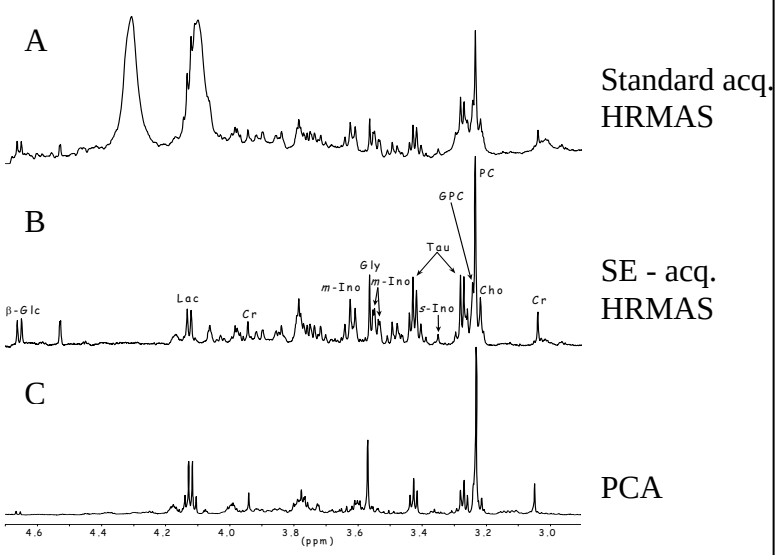


Figure 3: Spectral region 4.70 to 2.90 ppm from three different spectra of breast cancer tissue. A: MAS spectrum acquired using a standard acquisition, B: MAS spectrum from the same sample as in A recorded using a spin-echo sequence with 285 ms total echo time and C: spectrum of an breast cancer tissue extract. Some peak assignments are given in B, the following abbreviations are used: β-Glc; β-glucose, Lac; lactate, Cr; creatine, *m*-Ino; *myo*-inositol, Gly; glycine, Tau; taurine, *s*-Ino; *scyllo*-inositol, GPC; glycerophosphocholine, PC; phosphocholine and Cho; choline.

Data Interpretation

Different approaches are used to investigate HR MAS MR spectra. Spectral characteristics can be explored by examining peak intensities or peak areas. Peak areas can be obtained by integration or, in spectra where peaks are overlapping, by deconvolution (26). Peak-by-peak investigations to extract information have been useful in many studies, and makes direct comparison between chemical and biological features possible (3,4,27-29).

Quantitative determination of metabolite concentration in biological tissue is a challenge. Metabolites of interest can be quantified by comparing peak areas to an internal reference like water (30,31) or to an added reference (14,32,33). The method of referring the signals to internal water for calibration is not applicable in tissues with highly variable amounts of water. Added TSP as a reference has its limitations, as TSP can be associated to components in tissue, and thus induce decreased "NMR-visibility". Wu (33) and Taylor (32) have presented a method using a small silicon rubber within the sample volume for quantification. As the silicon rubber should not be in contact with the sample, this method is incompatible with sample preparations involving complete filling of the rotor volume. The ERETIC method (Electronic reference to access absolute concentrations directly) relies upon the generation of a defined frequency and amplitude by a broad-band antenna in the magnet of the MR-spectrometer, and thus provides an electronically synthesized reference signal (34). Such a method has yet to be implemented in combination with HR MAS.

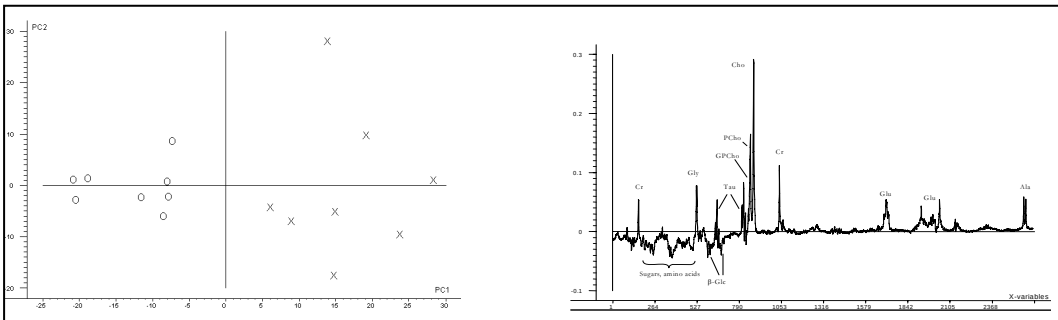
Spin-echo experiments are commonly used in MAS studies of biological samples to suppress broad resonances, and will cause a T_2 dependence of all metabolite signals that must be corrected for when quantifying the metabolites. This can be done by calculating individual T_2 for each peak and correct for the signal reduction of all the signals of interest. It is also important to ensure full T_1 relaxation in quantitative HR MAS experiments.

MR spectra from biological samples are often investigated with respect to a specific disease. Characterization of spectral findings from the disease can be attempted by comparisons between samples from different stages or to controls. Several MR spectroscopic studies have shown that almost all resonances influence the spectral patterns (35,36) and visual inspection of such spectra yield limited information from the available data. Recently, the term metabolomics has been introduced, defined as the complete set of metabolic components in a specific cell or tissue type, varying according to the physiological or pathological state of the system (37,38). Metabolomic strategies most often involve the combined use of multivariate statistics and an analytical technique. Multivariate data analysis is used for a number of purposes, which roughly can be divided into three groups: exploration, classification and prediction. The approaches where multivariate analysis is employed can be described as supervised and unsupervised.

- Unsupervised analysis is typically used for data exploration and classification, where samples are analyzed purely on the basis of the input variables, without the addition of previous knowledge. These input variables might be the relative intensities from a complete or selected region of spectra.
- Supervised analysis directly utilizes previous knowledge about patterns, groups or other related/measured variables. The purpose is often some kind of prediction, where the goal can be to predict class membership of future samples.

Principal component analysis (PCA) is a common unsupervised method (35,36,39,40). The objective of PCA is to convert the multiple and possibly correlated parameters from the measurements to a non-correlated and much smaller set of parameters (35). PCA creates

linear combinations from the original spectra based on the variance, leading to a reduced set of independent variables describing the original data set. The projections of the samples (individual spectra) onto the PCs are defined as scores, which reveal relationships between samples. In the graphic representation, called score plot, similar samples will group together in clusters. Another graphic representation useful for interpretation of the modeling is the loading profile, which connects the PCs to the original variables. Variables close to the origin in this plot carries little information in the PC, while variables with larger distance from the origin (high loading) are important in the interpretation.



A B

Figure 4: Score plot (A) and corresponding loading profile of PC1 (B) from principal component analysis (PCA) of the spectral region 4.1 – 1.4 ppm from spin-echo MAS spectra of biopsies from cervix from 16 patients. X denotes cervical cancer tissue, while O denotes cervix tissue from non-cancer patients. The cancer samples are clearly separated from the controls. The loading profile shows that the samples from patients with cervical cancer may be associated with higher concentrations of cholines, creatine, taurine and alanine. In addition, the cancer samples seem to have lower levels of glucose.

Soft independent modeling of class analogy (SIMCA) is a method for supervised pattern recognition (classification), and partial least squares regression (PLS), a related method for prediction. These methods will not be further discussed in this summary.

Artificial neural networks are electronic models based on the neural structure of the brain. The structure of a general neural network consists of nodes in three types of layers: input, hidden and output layer. (See structure of a probabilistic neural network in Figure 5)

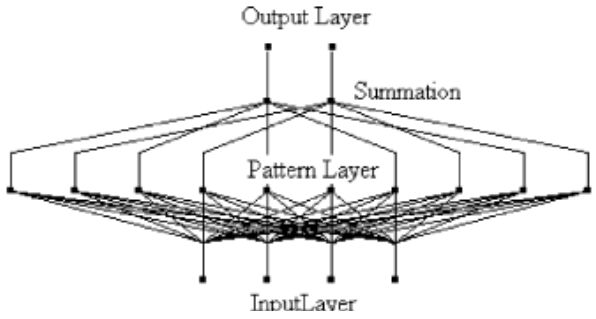


Figure 5: Structure of a probabilistic neural network

The number of input nodes will be equal to the number of input variables, and the number of output nodes depends on the number of outputs sought. Neural networks are able to detect and model nonlinear relationships, which may be an important property when working with complex data.

Current clinical research

One of the pioneer publications on HR MAS of human tissue was a study of human lymph nodes presented by Cheng and coworkers in 1996 (7). The HR MAS technique was shown to provide highly resolved spectra from intact tissue samples. It has later been applied in numerous types of tissue, and provided detailed descriptions of the chemical composition of healthy and affected tissue from kidney (41,42), brain (8,43) and prostate (18). A study of human kidney has provided classification of renal carcinomas (44) while MAS studies of brain tissue have shown that specific metabolites and metabolite ratios correlate to density of specific cell types (45) and fraction of cancerous and necrotic areas (46). Also in MAS studies of prostate tissue, metabolite concentration has been found to correlate to tissue composition (15,31). MAS MR spectra could also discriminate malignant prostate from healthy glandular tissue (15). A study on breast cancer tissue (47) has showed that breast carcinomas could be distinguished from non-involved breast tissue based on intensities and T_2 relaxation values of cellular metabolites. Recent work from our group shows that breast cancer biopsies can be classified according to tumor grade and lymph node status (14). Classifiers, which objectively could provide rapid and reliable information for determination of prognostic indicators simultaneously with the diagnosis of primary pathology and lymph node involvement, would obviously be beneficial.

The HR MAS technique is now also being explored as a tool for assessing treatment effects. Studies involving chemotherapeutic agents have been presented on cell lines (48), animal models (49-52) and tissue samples (53) in order to reveal molecular mechanisms and monitor treatment effects. HR MAS spectra have indicated binding between the drug hederacolchiside A1 to melanine in melanoma cell lines (48). Altered phospholipid mechanisms were found in a mouse melanoma model as response to chemotherapy (50,51). Gene-therapy induced apoptosis in BT4C rat gliomas has been characterized with increased levels of lipids and small metabolites (49,52). Altered phospholipid mechanisms have also been found in a study on liposarcoma cell line (53). In the same study, tissue samples from patients that received this drug were compared to samples from patients treated with surgery alone. This study indicates that HRMAS might have the potential of predicting good responders.

Future directions

HR MAS MR spectroscopy has been established as a valuable tool in cancer research. The clinical value of the method is not fully addressed, but clinical studies have been started. The results of such studies are important for the role of HR MAS MR spectroscopy as a method for providing clinical information on diagnostic and prognostic markers in cancer treatment.

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